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Development of Dual Control Allotopic Expression System for Subunit 8 of Yeast *Saccharomyces cerevisiae* Mitochondrial ATP Synthase

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The yeast mitochondrial F_1F_0-ATP synthase is a multisubunit complex that contains at least 17 different subunits. Subunit 8 of yeast mitochondrial ATP synthase is a hydrophobic protein of 48 amino acids encoded by the mitochondrial *ATP8* gene. A dual control allotopic expression system for subunit 8 has been developed. The strategy involves maintenance of two different compatible yeast expression vectors each utilizing a different inducible promoter in the same host cells. The system thus enables cloning and allotopic expression of two different forms of subunit 8 gene. The goal of the developed strategy is to permit allotopic expression of functional wildtype subunit 8 gene under a conditional promoter system and subunit 8 variant gene under the control of a different promoter system. The system is potentially useful for accurate assessment of assembly behavior of functionally defective subunit 8 variants *in vivo*. The strategy relies on the ability to conditionally regulate the expression of the two genes. A set of functionally defective subunit 8 variants has been cloned under an inducible yeast promoter system and dual plasmid harboring strains for dual control allotopic expression of each variant have been constructed.

Key words: expression system, ATP synthase, mitochondria, yeast

INTRODUCTION

The yeast mitochondrial F_1F_0-ATP synthase is a multisubunit complex that contains at least 17 different subunits. Subunit 8 of yeast mitochondrial ATP synthase is a hydrophobic protein of 48 amino acids encoded by the mitochondrial *ATP8* gene. Detailed information concerning the structure, function and topology of subunit 8 protein has been mainly derived from studies employing the allotopic expression system (Artika 2006). In this system, the subunit 8 gene is relocated to the nucleus and subunit 8 protein formed in the cytosol is targeted to the mitochondria. Following import into mitochondria, subunit 8 assembles into functional mitochondrial ATP synthase complex.

The yeast host cells employed in the allotopic expression of subunit 8 is the *mit*$^-$ M31 strain. This strain was derived from the wildtype J691B (MATα, *ade1*, *his6*, *rho*$^+$) (Macreadie *et al*. 1983) and carries a C to T base change at the position +7 of the *ATP8* gene. This mutation changes the third codon of *ATP8* gene to the stop codon TAA generating a predicted polypeptide of only two amino acids in length. Consequently, the M31 strain lacks endogenous subunit 8 protein and is unable to grow on nonfermentable carbon source such as ethanol or galactose due to lack of functional ATP synthase. Allotopic expression of functional form of subunit 8 gene rescues the ethanol negative phenotype of M31 strain. The functionality of subunit 8 variant, can therefore, be assessed by testing the ability of the allotopically expressed variant to restore the growth of M31 strain on ethanol medium (Artika 2006).

A number of yeast promoter sequences have been employed in the allotopic expression of subunit 8. Among them are the *PGK1*, *GAL1,* and *CUP1* promoter systems. Papakonstantinou *et al.* (1993) constructed a *PGK1* based yeast expression vector denoted pPD72 which has been extensively used for allotopic expression of subunit 8 variants. The *GAL1* based promoter system has also been used to achieve allotopic expression of subunit 8 gene under a tightly controllable promoter system. Law *et al.* (1990) employed the *GAL1* promoter system to investigate the *in vitro* assembly characteristics of allotopically expressed subunit 8. In these studies, the *GAL1* promoter was used to regulate expression of the subunit 8 gene such that subunit 8-depleted mitochondria capable of protein import could be generated for used in assessing the *in vitro* assembly characteristics of the allotopically expressed subunit 8 variants. Gray (1996) compared the level of allotopic expression of subunit 8 genes placed under the *CUP1* and *PGK1* promoter systems. It was found that high level expression of some subunit variants under transcriptional control of *CUP1* promoter led to improved growth rates compared to the corresponding variants expressed under the *PGK1* promoter.

The successful assembly of the nuclearly expressed subunit 8 in this system has been confirmed by immunoadsorption assays using an anti-subunit β antibody (Nagley *et al.* 1988). The immunoadsorption assays is also used in *in vitro* system developed for

detecting subunit 8 assembly from complexes isolated from yeast mitochondria (Law & Nagley 1990; Papakonstantinou *et al*. 1993). This system, while useful in studying the assembly of defective mutants which cannot grow on respiratory substrates, it may not always faithfully reflect the *in vivo* assembly behavior of a particular variant protein. This phenomenon is illustrated by subunit 8 variant (K47STP) which was functional *in vivo* but showed little assembly based on data obtained from *in vitro* assays (Gray 1996).

One of the problems in testing the assembly of non-functional or functionally impaired variants is the tendency of the *mit*$^-$ mutant host cells to turn into petite cells. It has been observed that yeast cells with non-functional mitochondria are genetically unstable and they have a tendency to lose an introduce plasmid (Gray 1996). It is important, therefore, to design an *in vivo* system for assessing the assembly of functionally defective mutants. The present study deals with development of an expression system termed dual control allotropic expression system with potential application for testing the assembly of functionally impaired subunit 8 variant *in vivo*. The strategy involves maintenance of two different yeast expression vectors each utilizing a different promoter thus enabling allotopic expression of two different forms of subunit 8 gene in the same *mit*$^-$ host cells.

MATERIALS AND METHODS

Materials. *Saccharomyces cerevisiae* strain M31 [*atp8*, mit$^-$, *his*6, *ade*1], a collection strain of the Department of Biochemistry and Molecular Biology, Monash University, has previously been described (Nagley *et al*. 1988). Strain YGL1 (provided by Dr. RHP Law) is strain M31 expressing subunit 8 gene cloned under the *GAL1* promoter in vector pED121 (Law *et al*. 1990). Plasmid pRD643 is plasmid vector pGEM7Zf(+) harboring *HIS6* gene (Fani *et al*. 1997). Plasmid pYEUBLX is a yeast expression vector employing *CUP1* promoter system (Macreadie *et al.* 1991). The set of genes encoding subunit 8 variants with mitochondrial signal peptide has been described (Papakonstantinou *et al.* 1996; Artika 2007).

DNA Modification and Molecular Cloning. The restriction site *Apa*I was introduced into the pRD643 plasmid by using PCR-based mutagenesis technique. The primer used (5'GCTCTCCGGGCCCGAACTGACTTTC3') was designed to introduce *Apa*I restriction site (5'GGGCCC3') into the multiple cloning site of pRD643. PCR was carried out for 30 cycles. The PCR products were analyzed by agarose gel electrophoresis technique. Cloning of DNA fragment into the yeast expression vector was carried out using standard methods (Sambrook *et al*. 1989).

Construction of Yeast *CUP1* Vector Carrying *HIS6* Selectable Marker. The construction of *HIS6* containing-yeast *CUP1* vector was based on a multicopy vector bearing a *CUP1* promoter cassette, the pYEULBX (Macreadie *et al*. 1991). The *HIS6*/*CUP1* vector was constructed by retrieving a *HIS6* gene contained in the plasmid pRD643 (kindly provided by Dr. Renato Fani; Fani *et al*. 1997) into the plasmid vector pYEULBX. Following introduction of an *Apa*I site into the PRD643 using PCR-based mutagenesis technique, the PCR products were then purified and digested with *Aat*II and *Apa*I restriction enzymes. The digestion products were separated in agarose gel electrophoresis and the band corresponding to the HIS6 *Aat*II/*Apa*I DNA fragment was excised from the gel. Following recovery and purification, the DNA fragment was ligated with pYEULBX plasmid previously cut with *Aat*II and *Apa*I restriction enzymes. To examine the successful introduction of the *HIS6* gene into the pYEULBX, a set of putative recombinant DNA plasmids was isolated and digested with *Aat*II and *Apa*I restriction enzymes followed by analysis of digestion products using electrophoresis technique.

Cloning of Subunit 8 Variants Into the pAD1. Cloning of subunit 8 variant into the pAD1 plasmid vector was carried out by inserting a DNA segment containing subunit 8 variant gene into the cloning site of pAD1. A fragment containing subunit 8 variant was generated by digesting a pGEM-T vector containing the corresponding variant with *Bam*HI and *Not*I restriction enzymes. The digestion products were then separated on agarose gel electrophoresis. DNA corresponding to subunit 8 variant gene was excised from the gel, purified and then ligated into the pAD1 previously cut with *Bam*HI and *Not*I enzymes. The ligation mixture was then introduced into *E. coli* cells and followed by selection of transformants. Positive clones were screened by isolating plasmid DNA followed by restriction enzyme disgestion analysis using *Bam*HI and *Not*I enzymes in order to confirm the successful introduction of subunit 8 variant gene into the pAD1 plasmid vector.

Construction of Dual Plasmid Harboring Strain. Dual plasmid containing strains were created by introducing the pAD1 vector into YGL1 cells (M31 strains harbouring pED121 vactor) by using yeast transformation procedure (Klebe *et al*. 1983). The pED121 plasmid vector harbors *URA3* and *ADE1* genes as selectable markers. The pAD1 vector harbors *HIS6* gene as a selectable marker. To select cells carrying both pED121 and pAD1 recombinant plasmids, the transformants were plated on solid minimal medium without amino acid supplementation.

RESULTS

Yeast *CUP1* Vector Carrying *HIS6* Selectable Marker. The yeast *CUP1* vector carrying *HIS6* selectable marker was constructed by introducing a *HIS6* gene into the plasmid vector pYEULBX. The successful introduction of the *HIS6* gene into the pYEULBX was shown by release of a fragment of about 1.5 kb (Figure 1) following digestion of a set of putative recombinant DNA plasmids with *Aat*II and *Apa*I restriction enzymes. The resulted plasmid vector was then denoted pAD1.

Subunit 8 Variants Cloned Into the pAD1. Cloning of subunit 8 variant into the pAD1 plasmid vector was carried out by inserting a DNA segment containing subunit 8 variant gene into the cloning site of pAD1. Successful introduction of the gene into the pAD1 plasmid was

indicated by release of a fragment of 641 bp (Figure 2) following digestion of a set of putative recombinant plasmids by using *Bam*HI and *Not*I enzymes. In the present study, a wildtype subunit 8 gene and four different subunit 8 variant genes were cloned into the pAD1 vector (Table 1). Each of the variant contains FLAG epitope tag (DYKDDD) at its C-terminus.

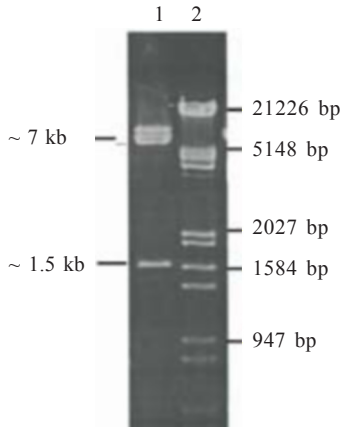

Figure 1. Restriction enzyme digestion analysis of recombinant plasmid pAD1. The pAD1 vector was constructed by inserting a DNA fragment of about 1.5 kb harboring *HIS6* gene into plasmid pYEULBX. The pAD1 vector construct was analyzed by restriction enzyme digestion using *Aat*II and *Apa*I restriction enzymes. The digestion products were then separated on agarose gel electrophoresis. Lane 1: *Aat*II/*Apa*I digest of putative plasmid pAD1. The upper band in this lane is the segment of vector pYEULBX of about 7 kb. The 1.5 kb band is the DNA sement containing the *HIS6* gene; Lane 2: lambda DNA digested with *Hind*III and *Eco*RI. The size of several fragments is indicated (bp).

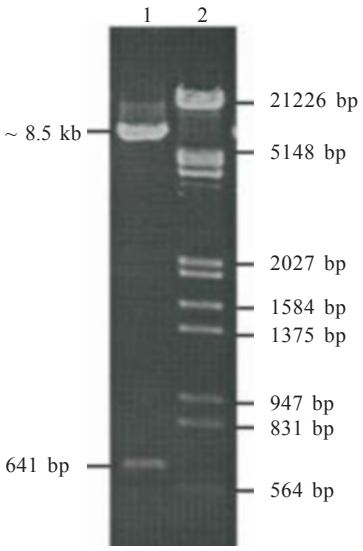

Figure 2. Restriction enzyme digestion analysis of recombinant plasmid pAD1::subunit 8 variant. The pAD1::subunit 8 vector was constructed by inserting a DNA fragment of about 641 bp harboring subunit 8 gene into plasmid pAD1. The resulted recombinant plasmid was analyzed by restriction enzyme digestion using *Bam*H1 and *Not*I restriction enzymes. The digestion products were then separated on agarose gel electrophoresis. Lane 1: *Bam*HI/*Not*I digest of putative recombinant plasmid pAD1::subunit 8 variant. The upper band in this lane is the segment of vector pAD1 of about 8.5 kb. The 641 bp band is the subunit 8 variant gene segment as expected. Lane 2: lambda DNA digested with *Hind*III and *Eco*RI. The size of each fragment is indicated (bp).

Dual Plasmid Harboring Strain. The dual plasmid harboring strain was created by introducing the pAD1 vector harboring subunit 8 variant gene into the YGL1 cells. Results showed that the pAD1 plasmid carrying each of the variant was successfully introduced into YGL1 cells as shown by their growth on selective minimal medium without amino acid supplementation (Figure 3). List of subunit 8 variants cloned into pAD1 and the dual plasmid harboring strains generated is presented in Table 1.

DISCUSSION

Gray (1996) found that subunit 8 gene expressed under the *CUP1* promoter system can only restore the growth of M31 cells on non-fermentable substrate if the vector is a multicopy vector. It was concluded that the copy number of the vector is crucial in dictating whether the level of subunit 8 expression is enough to generate functional ATP synthase complex. For these reasons, in the present study, the construction of *HIS6* containing-yeast *CUP1* vector was based on a multicopy vector bearing a *CUP1* promoter cassette, the pYEULBX (Macreadie *et al*. 1991).

Table 1. Yeast strains harboring two recombinant plasmids for dual control allotopic expression of functionally defective subunit 8 variant gene

Strain	Recombinant plasmids*
YMA30	pED121::Y8 (wildtype) + pAD1::Y8 (wildtype)
YMA68	pED121::Y8 (wildtype) + pAD1::Y8 (L23D, L24D, DYKDDD)
YMA70	pED121::Y8 (wildtype) + pAD1::Y8 (Q29D, F30D, DYKDDD)
YMA71	pED121::Y8 (wildtype) + pAD1::Y8 (Q29R, F30R, DYKDDD)
YMA23	pED121::Y8 (wildtype) + pAD1::Y8 (L23P, DYKDDD)

*Y8 = subunit 8 gene.

Figure 3. Yeast strains harboring two recombinant plasmids for dual control allotopic expression of functionally defective subunit 8 variant gene. Each of the yeast strain YMA30, YMA68, YMA70, YMA71, and YMA23 was generated by transforming strain YGL1 with plasmid pAD1 harboring different subunit 8 variant gene. Transformant cells were then grown on solid minimal medium without amino acid supplementation. The growth of the transformants on this medium indicated that the transformation process was successful.

There was a problem associated with the strategy for introducing *HIS6* marker into pYEULBX in that there was no compatible restriction sites by which the *HIS6* gene could be excised from pRD643 and then inserted into pYEULBX. In order to overcome this problem, an *Apa*I site was introduced using oligonucleotide-directed mutagenesis into the multiple cloning sequence region of pRD643. This allowed recovery of the *HIS6* segment as an *Aat*II/*Apa*I fragment which could then be conveniently inserted into pYEULBX previously cut with the same enzymes.

The ultimate goal of the dual control allotopic expression system is to permit allotopic expression in *mit*$^-$ M31 cells of functional (wildtype) subunit 8 under a conditional promoter system and subunit 8 variant under the control of a different promoter system. The expression of wildtype subunit 8 gene is intended to ensure the maintenance of stable *mit*$^-$ yeast cells during cell propagation. It is under conditional expression control so that its expression can be turned off and then the expression of the variant can be turned on and its assembly behavior determined. The strategy relies on the ability to conditionally regulate the expression of the two genes.

In the developed strategy, the wildtype subunit 8 gene will be allotopically expressed under the control of the inducible *GAL1* promoter system incorporated in a yeast expression vector (pED121) carrying *ADE1* gene as a selectable marker. The non-functional (or functionally impaired) subunit 8 variant will be allotopically expressed under the control of the *CUP1* promoter system incorporated in a second yeast expression vector (pAD1) carrying the *HIS6* gene as a selectable marker. The functionality the *HIS6* gene as a selectable marker for yeast vector has previously been demonstrated (Artika 2009).

Application of the basic strategy to regulate the expression of the two genes can be achieved as follows. In the presence of galactose in the growth medium, transcription from the *GAL1* promoter is induced and the functional subunit 8 gene will be expressed. The biosynthesis of subunit 8 in the cytosol, its import into mitochondria followed by successful assembly into the mitochondrial ATP synthase complex will ensure the maintenance of stable yeast cells. Under these conditions, the subunit 8 variant gene might well also be expressed (by virtue of small amount of Cu_2^+ in the medium), however the subunit 8 protein assembled into the enzyme complexes would be predominantly the wildtype version.

Removal of galactose from the growth medium, and at the same time addition of Cu_2^+ into the medium, would stop the expression of the functional subunit 8 and lead to strong expression of the subunit 8 variant gene. The ability of the overexpressed subunit 8 variant to assemble into functional mitochondrial ATP synthase complex can now be assessed. In practice this can be performed by immunological analysis to detect the presence of the subunit 8 variant in the assembled complex. For instance, the mitochondrial ATP synthase complex can be immunoprecipitated using an anti-subunit β antibody (Nagley *et al*. 1990). Detection of the subunit 8 variant can also be facilitated by attaching a peptide reporter such as the FLAG epitope tag to the subunit 8 variant protein. All of the variants used in the present study incorporate FLAG epitope tag at the C-terminus. Previous study has shown that the FLAG tagged-subunit variants can efficiently be detected in a Western blot analysis using the anti-FLAG M2 monoclonal antibody (Artika 2006).

By applying the developed dual control allotopic expression system, mutations affecting the assembly capacity of subunit 8 can potentially be distinguished from mutations affecting subunit 8 function in the enzyme complex. If the failure of a variant protein to display function is due to the inefficient assembly of the variant subunit into the enzyme complex, overexpression of the particular subunit 8 variant under *CUP1* promoter control would improve the cellular function of the enzyme complex which can be determined by measurement of generation time. To the contrary, if the variant does not affect the assembly process but rather affects the function of the subunit 8, overexpression of the particular variant under *CUP1* promoter control is not expected to result in functional improvement.

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